AD_____

AWARD NUMBER: W81XWH-05-1-0081

TITLE: Identifying Molecular Targets for Chemoprevention in a Rat Model

PRINCIPAL INVESTIGATOR: Ralph W. deVere White, M.D.

CONTRACTING ORGANIZATION: University of California, Davis
                                          Davis, California   95616-8671

REPORT DATE: December 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-12-2005	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 15 Nov 2004 – 14 Nov 2005
4. TITLE AND SUBTITLE Identifying Molecular Targets for Chemoprevention in a Rat Model		**5a. CONTRACT NUMBER**
		5b. GRANT NUMBER W81XWH-05-1-0081
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Ralph W. deVere White, M.D. E-Mail: rwdeverewhite@ucdavis.edu		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, Davis Davis, California 95616-8671		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this grant is to determine the molecular events that occur in the dorsal and ventral lobes of the rat prostate gland after 20 weeks of exposure to PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridene). PhIP is a potent inducer of mutations in the rat prostate where we have shown that it forms bulky DNA adducts. In addition, it induces high levels of oxidative damage in the target tissues. The scope of this research includes: 1) Generation of a rat model, 2) Analysis of the rats prostate after 20 weeks of PhIP, and 3) Gene chip microarray analysis. To date, we have completed the histopathologic analysis of PhIP induced prostate pathology, we have generated an inflammatory/atrophy/proliferation model, and we have acquired data that refutes a previous model of neoplastic progression. These results have been summarized in a paper submitted to the journal Neoplasia.

15. SUBJECT TERMS
PhIP (2-amino-1-methyl-6phenlimidazo[4,5-b]pyridine) Prostate Cancer, Rats, Chemoprevention

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	27	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

　　　　1) Inflammation and Atrophy Precede Prostate Neoplasia
　　　　　　in PhIP Induced Rat Model. Submitted to *Neoplasia*, May 2006.

Title: Identifying Molecular Targets for Chemoprevention in a Rat Model.
Principal Investigator: Ralph W. deVere White, MD
PC040947 (Contract # W81XWH-05-1-0081)

INTRODUCTION: The **subject** of this DOD grant is to create a chemoprevention strategy for Prostate Cancer (aP) because of its high incidence and long natural history. The NCI has undertaken two large chemopreventive clinical trials, the Prostate Cancer Prevention Trial (PCPT) and the Selenium and Vitamin E Cancer Prevention Trial (SELECT). These combined trials accrued over 40,000 men. We believe that what is missing in studies of the chemoprevention of CaP is an animal model with well-defined molecular characteristics. Using a well-characterized animal model would permit rapid proof-of-principle pre-clinical chemoprevention studies to be performed. The **purpose** of this grant is to determine the molecular events that occur in the dorsal and ventral lobes of the rat prostate gland after 20 weeks of exposure to PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridene). PhIP is a potent inducer of mutations in the rat prostate where we have shown that it forms bulky DNA adducts. In addition, it induces high levels of oxidative damage in the target tissues. The **scope of this research** includes: 1) Generation of a rat model, 2) Analysis of the rats prostate after 20 weeks of PhIP, and 3) Gene chip microarray analysis

BODY:

Research accomplishments associated with:

1) <u>Generation of a rat model</u> – Male Fischer F344 Rats (Simonsen Laboratories, Gilroy,CA) were housed under the care of Lawrence Livermore Animal facility and treated per guidelines outlined in this DOD grant. Forty rats at 5 weeks of age were fed PhIP in the diet for 20 weeks. For the first 13 weeks, PhIP was fed to the animals at 400ppm in a certified basal diet. However, due to weight loss in the animals, the PhIP content was reduced to 200 ppm for the final 7 weeks. Forty controls received basal diet without the addition of PhIP. At 20 weeks, 10 PhIP-dosed and 10 control animals were euthanized.

2) <u>Analysis of the prostate after 20 week of PhIP</u> - Inflammation in the PhIP treated rat prostate was the earliest, and most obvious change as compared to the control group (Figure 1a). Inflammation was found in all prostatic glands, but was least prominent in the anterior or coagulating gland. Inflammation persisted after discontinuing the PhIP treatment, and epithelial layers at all of the scheduled time points were intact, even in areas of luminal microabcess. Many of the areas of inflammation were accompanied by a reactive stromal proliferation resulting in a distinct thickening of the thin muscular layer surrounding individual glands. These proliferations did not appear to over grow the reactive process or to become neoplastic, as has been reported in some mouse models of prostate cancer [1].

Large areas of glandular atrophy, particularly affecting the ventral prostate were seen in all treated animals. Non-treated animals also appeared to be prone to glandular atrophy, particularly in the ventral prostate, but with less area of the prostate involved (Figure 1b).

No invasive carcinoma was seen in any of the animals. There were areas of inflammation induced stromal proliferation and high levels of inflammatory atypia. Not all of the animals were examined, but of the 20 animals examined at least with segmental histology of the intestine, only one had a full fledged polypoid adenoma and only two others had areas of early adenomatous changes.

Figure 1.

Gland Inflammation

Gland Atrophy

Gland Atypia

Gland PIN
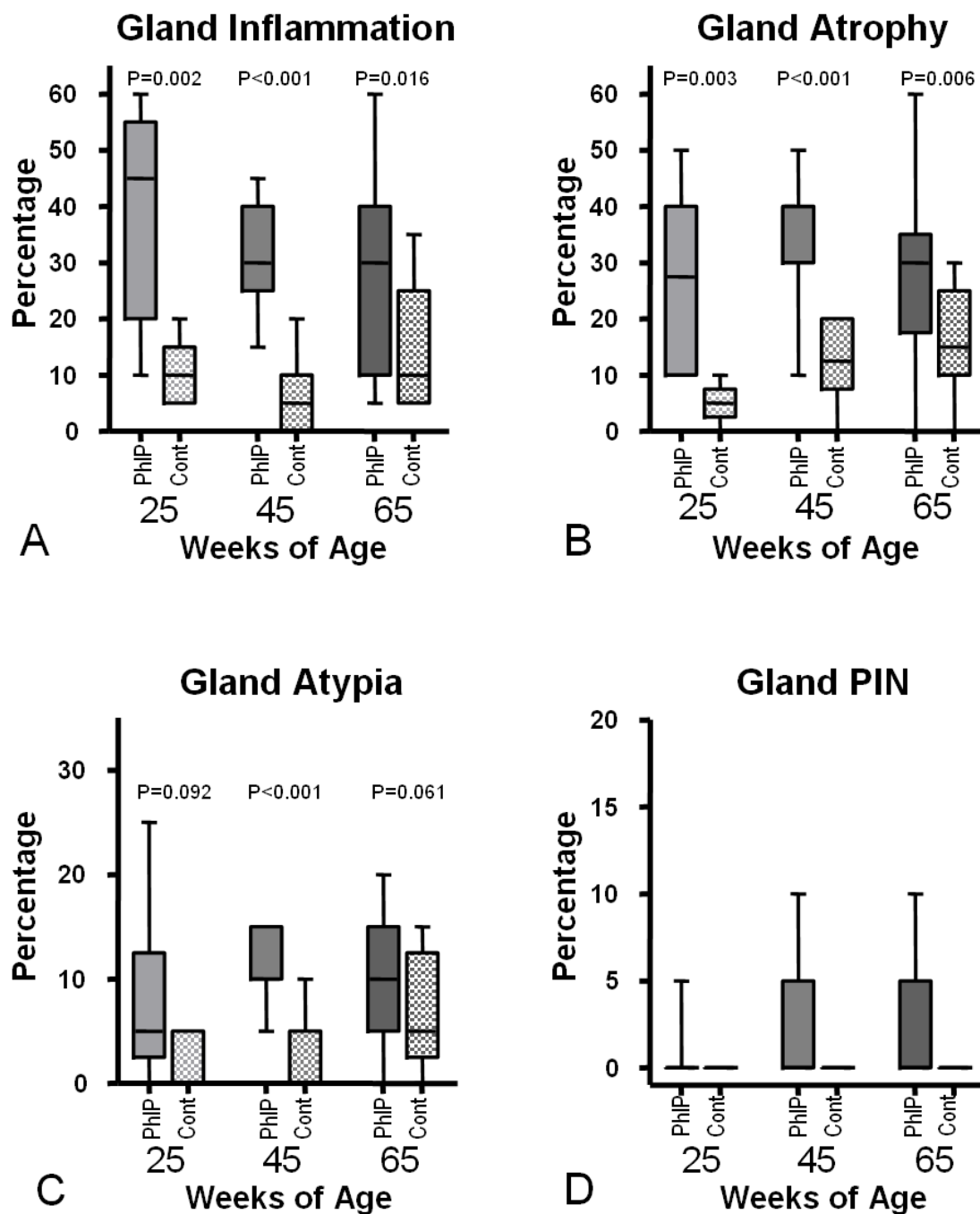
3) Gene chip microarray analysis – Initially we planned to dissect subgross lesions in the prostate identified by surgical microscopy. Unfortunately the PIN lesions we eventually identified by histologic processing and transmitted light microscopy were too small to be seen in the surgical microscope. Furthermore, the amount of tissue/amount of RNA present in these

5

foci would require analysis compromising amplifications to be used in Affymetrix arrays. We have modified the aim to evaluate the intermediate conditions of inflammation and atrophy in the prostate by gene expression analysis. Because this analysis involves multiple cell types and glands in various and heterogeneous stages of inflammation, proliferation, atrophy and neoplasia, we anticipate a good deal of insensitivity and non-specificity. Nevertheless, candidate genes identified in this way will be subsequently evaluated using immunohistochemical analysis of protein expression. This may provide semiquantitative data, but also provides excellent cell and tissue localization of protrein expression. These studies are still underway.

During the execution of the second year of this DOD grant, Dr. Karen Dingley from Lawrence Livermore National Laboratory (Consultant) accepted a position with another institution and departed. Although she continued her collaborative efforts on the manuscript generated from this grant, she was not directly involved with the ongoing care of the rats at LLNL. The departure of Dr. Dingley impacted our original timeline from the actual treatment of the rats with PhIP to the analysis phase by Dr. Borowsky.

KEY RESEARCH ACCOMPLISHMENTS:

- **Histopathologic analysis of PhIP induced prostate pathology**
- **Generation of an inflammatory/atrophy/proliferation model**
- **Data generated which refutes a previous (more simplistic) model of neoplastic progression.**
- **Data generated which calls into question some previously reported findings.**

REPORTABLE OUTCOMES:

Inflammation and Atrophy Precede Prostate Neoplasia in PhIP Induced Rat Model. Borowsky AD, Dingley K, Ubick E, Turteltaub K, Cardiff RD, deVere White RW. Submitted to Neoplasia, May 2006.

CONCLUSIONS:

The importance of this research is that it has shown that the PhIP treated rat can be a useful model of human disease suitable for testing prevention strategies targeting either DNA adduct formation or prostate inflammation as potential initiators and promoters of prostate cancer progression. The implication of this work will lead to the ability to design successful chemopreventive strategies so that the cancer either does not develop, or takes a far longer time to transition from a pre-clinical to a clinical stage. This research has the potential to spare many patients from having to deal with this disease.

REFERENCES:

1. Shappell SB, Thomas GV, Roberts RL, Herbert R, Ittmann MM, Rubin MA, et al. Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res 2004;64:2270-305.

APPENDICES:

Inflammation and Atrophy Precede Prostate Neoplasia in PhIP Induced Rat Model. Borowsky AD, Dingley K, Ubick E, Turteltaub K, Cardiff RD, deVere White RW. Submitted to Neoplasia, May 2006.

Inflammation and Atrophy Precede Prostate Neoplasia in PhIP Induced Rat Model

Running Title: PhIP Rat Prostate

Alexander D. Borowsky[1]*, Karen Dingley[2], Esther Ubick[2], Kenneth W. Turteltaub[2], Robert D. Cardiff[1], Ralph DeVere-White[3]

UC Davis
[1]Center for Comparative Medicine
Department of Medical Pathology
UC Davis School of Medicine
Davis, CA 95616
adborowsky@ucdavis.edu
530-754-8182, fax 530-752-7914

[2]Biosciences Directorate
Lawrence Livermore National Laboratory
Livermore, CA 94550

[3]Comprehensive Cancer Center
Department of Urology
UC Davis School of Medicine
Sacramento, CA 95817

*To whom correspondence should be addressed.

Abstract

2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) has been implicated as a major mutagenic heterocyclic amine in the human diet and is carcinogenic in the rat prostate. In order to validate PhIP induced rat prostate neoplasia as a model of human prostate cancer progression, we sought to study the earliest histologic and morphologic changes in the prostate and to follow the progressive changes over time. We fed 67 male Fischer F344 5 week old rats with PhIP (400 PPM) or control diets for 20 weeks, and then sacrificed animals for histomorphologic examination at age 25 weeks, 45 weeks, and 65 weeks. Animals treated with PhIP showed significantly_more inflammation (P=.002 (25wk), >.001(45wk), .016(65wk)) and atrophy (P=.003(25wk), >.001(45wk), .006 (65wk)) in their prostate glands relative to controls. Prostatic intraepithelial neoplasia (PIN) occurred only in PhIP treated rats. PIN lesions arose in areas of glandular atrophy, most often in the ventral prostate. Atypical cells in areas of atrophy show loss of glutathione S-transferase pi immunostaining preceding development of PIN. None of the animals in this study developed invasive carcinomas differing from previous reports. Overall, these findings suggest that the pathogenesis of prostatic neoplasia in the PhIP treated rat prostate proceeds from inflammation to post-inflammatory proliferative atrophy to PIN.

2

Introduction

Prostate cancer is the second leading cause of cancer death for men in the US. Specific etiologies remain unknown, but there have been associations with dietary factors such as the consumption of red meat and saturated fat (ref). Interestingly, prostate cancer rates are extremely low in Asia, particularly in China (ref). While it is not known whether inherited or environmental factors are primary, several associations have been made.   Emigrants from eastern countries to the west approach western rates for prostate cancer within one generation, potentially correlating with adoption of a "western diet"[1] Autopsy studies of American men and Chinese men show a distinct difference in prostatitis with extremely high rates in Americans and essentially no inflammation in Chinese[2-4].   Several experimental associations recently have been made between prostatic inflammation and prostate cancer risk[5, 6].

2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) has been implicated as a major mutagenic heterocyclic amine in the human diet[7]. We have shown that PhIP forms DNA adducts in prostate epithelial cells, and induces mutations in rat prostate, mammary gland, and intestines when administered experimentally[8].

It has been reported that roughly 50% of Fischer F344 male rats treated with PhIP at one year of age have invasive prostate carcinoma[9]. No report of earlier pathologic lesions or associated gland changes has been produced. The images of the histology of the PhIP induced prostate cancers are hard to compare to the histomorphology of human prostate carcinoma.   In order to characterize the progression of prostate neoplasia, and to compare the morphology and biology of the PhIP induced prostate cancers to human disease, we sought to study early and intermediate time points in these animals. We also sought to study control animals at the same ages and housed under identical conditions.

Methods

Animal Treatment

Male Fischer F344 Rats (Simonsen Laboratories, Gilroy, CA) were housed at the AALAC-accredited Lawrence Livermore National Laboratory Animal Care and Use facility and treated according to the guidelines in the "Guide for Care and Use of Laboratory Animals (Laboratory Animal Resources, National Research Council) with Institutional Animal Use and Care Committee approval. Forty rats at 5 weeks of age were fed PhIP (Toronto Research Chemicals, Ontario, Canada) in the diet for 20 weeks. Animals were weighed weekly throughout the study. For the first 13 weeks, PhIP was fed to the animals at 400ppm in a certified basal diet (Harlan Teklad Global 18% protein rodent diet). However, due to weight loss in the animals, the PhIP content was reduced to 200ppm for the final 7 weeks. Forty controls received basal diet without the addition of PhIP. At 20 weeks, 10 PhIP-dosed and 10 control animals were euthanized. Their urogenital and other grossly abnormal tissues were isolated and fixed in 10% neutral buffered formalin for histological examination. The remaining animals were all returned to a regular basal diet with euthanasia and histological examination at 40 and 60 weeks after treatment (45 and 65 weeks of age). Historically, it is reported that over 50% of animals have invasive tumors at 60 weeks [10]. The purpose of using the intermediate time points, as well as careful examination of the non-tumorous rats at 65 weeks, was to characterize the pre-cancerous histomorphology.

Histology

Necropsy tissues including the prostate, urethra, bladder, seminal vesicles, testes and bulbourethral glands were collected. Additional tissues were collected where possible, including intestines, lungs, liver, kidney, pancreas. All tissues were fixed overnight in neutral buffered formalin and then transferred to 70% ethanol, before processing. Tissues were paraffin embedded using standard histology protocols and sectioned at 4 microns. The resulting sections were stained with hematoxylin and eosin (H & E) and examined under light microscopy. Tissue blocks containing the majority of the tissues of the prostate, particularly ventral, dorsal and lateral glands, were identified for serial step sectioning. Additional levels through these blocks were mounted on + coated slides (Fischer) with 5 alternating levels stained with H&E for morphologic analysis.

Immunohistochemistry

Immunohistochemistry was performed on the 4 micrometer sections mounted on

+ coated slides. Slides were incubated at 100°C in 10mM Citrate , for antigen retrieval. Primary antibodies were incubated overnight at room temperature in PBS. Primary antibodies were used as follows: Ki-67 1:1000(LabVision), GSTP1 1:400 (Calbiochem, 354211), Cox2 1:250 (Calbiochem,236004). Secondary antibodies and detections were performed using the biotinylated secondary antibody and the EnVision HRP kit (DAKO, Carpenteria, CA) following

4

the manufacturers instructions. Histologic images were captured on a Zeiss Axioskop light microscope and photographed with the Zeiss AxioCam digital camera.

Quantitation
The serial step sections of ventral, dorsal, lateral, and sections of anterior prostate glands were examined blind to treatment group. In each, the total volume of any gland presenting inflammation, atrophy, atypia, or prostatic intraepithelial neoplasia was estimated by adding and averaging the slide surface areas involved by these characteristics. In general, percent gland affected was given to the nearest 5%. Inflammation was scored if there was luminal abscess or if there was evident peri- or intra- epithelial infiltration of any inflammatory cells (mononuclear or segmented neutrophils). Often this was accompanied by an increased surrounding stromal thickness. Atrophy was defined as a flattened epithelial cell lining with individual cells displaying a greater lateral width than basal to apical height. Areas of atypia were defined as foci of cells with increased nuclear size and hyperchromasia in the absence of inflammation. There were areas of atypia directly associated with inflammation, but these were not counted in the quantitative analysis. Areas of prostatic intraepithelial neoplasia (PIN) were defined as multiple cell layers with loss of cellular polarity and with evidence of focality and proliferation. Areas of proliferation remaining in a single cell layer were not defined as PIN.

Statistical analysis was performed using Graph Pad Prism software version 4.0.3.   An unpaired non-parametric t-test was applied to each data set for each of the quantitated characteristics. The resulting p values are recorded above each pair of data sets compared, and the data is represented with range, 25th-75th percentiles, and medians plotted as shown in Figure 1.

Results
Animal Treatment
During the study, the PhIP-dosed rats consistently gained less weight than the control animals. After 13 weeks of dosing at 400ppm, the rats began to lose weight. Consequently, the PhIP dose was reduced to 200ppm for the remaining 7 weeks of dosing. After PhIP treatment was complete, the growth rate of the PhIP-treated animals was the same as the controls, but these rats remained smaller than controls. At week 20, at the end of treatment, the mean body weights of PhIP dosed animals were 191 (+/-30) grams compared to controls which were 413 (+/-25) grams. By week 40, the treated rats had gained slightly more than controls, with mean body weights of 287 (+/-42) grams compared to controls 479 (+/-25) grams; and by week 60, the PhIP-dosed rats appeared to have the same mean growth rate as the untreated controls, though they did not "catch up" or grow at an accelerated rate (336 (+/- 39) grams for treated compared to 525 (+/- 37) grams for control). In addition, 10 PhIP-dosed animals and two control animals died or were euthanized due to deteriorating health prior to the termination of the study.

Prostate Histomorphology
Inflammation in the PhIP treated rat prostate was the earliest and most obvious change, as compared to the control group (Figure 1a). In the one rat that died during PhIP treatment, the prostatic epithelium was discontinuous with areas of epithelial loss or denudement. These areas were accompanied by inflammation and luminal debris. Inflammation was found in all prostatic glands, but was least prominent in the anterior or coagulating gland. Inflammation persisted after discontinuing the PhIP treatment, and epithelial layers at all of the scheduled time points were intact, even in areas of luminal microabcess. Many of the areas of inflammation were accompanied by a reactive stromal proliferation resulting in a distinct thickening of the thin muscular layer surrounding individual glands. These proliferations did not appear to overgrow the reactive process or to become neoplastic, as has been reported in some mouse models of prostate cancer[11]. Inflammation was seen focally in many of the control animals, but involved fewer glands, and was generally more mild. Where the experimental animals had dense exudative luminal content, the control animals had only mild exudates with scattered luminal neutrophils.

Large areas of glandular atrophy, particularly affecting the ventral prostate were seen in all treated animals. Non-treated animals also appeared to be prone to glandular atrophy, particularly in the ventral prostate, but with less area of the prostate involved (Figure 1b). Interestingly, only in the treated animals was proliferation seen interspersed within areas of atrophy, and the PIN lesions seen in the later scheduled sacrificed animals (45 and 65 weeks) occurred in areas of atrophic ventral prostate in the majority of cases (Figure 4).

PIN lesions seen in the PhIP treated rats were characterized by a cribriform architecture with well differentiated epithelial cells forming solid bridges and

6

circular apolar lumina. The lesions filled the gland lumen, but did not show distension with foci of stromal interruption. These features would be at least grade 2 (of 4) and most would be grade 3 (of 4) in published grading criteria for PIN in rodents [12].

No invasive carcinoma was seen in any of the animals. There were areas of inflammation induced stromal proliferation and high levels of inflammatory atypia (atypia of repair). Consistent with the low rate of prostate neoplasia, we also observed a lower than expected rate of intestinal neoplasia. Not all of the animals were examined, but of the 20 animals examined at least with segmental histology of the intestine, only one had a full fledged polypoid adenoma, and only two others had areas of early adenomatous changes.

Immunohistochemistry
Ki67 staining confirmed a high rate of proliferation in the areas of PIN with a percentage nuclear positivity in these areas of 15-20%. All other areas of the glands showed a range from 1-2% in normal non-inflamed areas to 5-10% focally in areas with inflammation (Figure 2B) or areas without inflammation but with aberrant cytologic features (Figure 3). This is consistent also with the finding of mitotic figures in these foci. There was no significant increase in apoptosis in these areas.

GSTpi immunostaining confirmed that the PIN lesions, specific cell populations in the areas of atrophy, and focal areas with atypical cells also showed loss of GSTpi (Figure 5). The precise relationship of the GSTpi negative atypical cells and the development of PIN was difficult to estimate. The prostate samples did not show the two populations in continuity, nor was there a continuous spectrum of lesions in between the polarized single layer of atypical cells to the fully developed PIN lesions. Cox2 levels were variable, but no cell foci appeared completely negative.

Leydig Cell Tumors
All rats in the 65 week group (both treated and controls) showed testicular Leydig cell hyperplasias with several in both groups with very large areas that might be regarded as Leydig cell tumors (Figure 6). These were characterized by well differentiated Leydig cells of the testicular stroma with abundant cytoplasm and focal crystalloid like cytoplasmic inclusions. This is a common finding in Fischer rats[13]. The testicular tubules were normal with good maturation of germ cells. The amount of testosterone production and serum testosterone levels is not known. A few animals in both the treated and control groups at 45 weeks had focal mild Leydig cell hyperplasia. Animals in the 25 week group had no evidence of testicular pathology.

Discussion

For the first time, we have carefully examined the prostate pathology of the precancer stages in the PhIP rat model. In our studies it seems clear that PhIP is capable of inducing a rapid and persistent prostatic inflammation. The mechanism of inflammation induced by PhIP is not precisely clear, but appears to involve a specific toxicity to the prostatic epithelium. This may result in an immediate disruption of the epithelial barrier and subsequent inflammation. However, this does not adequately explain the persistence of the inflammation. In some areas without inflammation an atypical proliferation is seen. In areas near inflammation, this is similar to the epithelial reaction seen in any inflamed mucosa. The areas that continue to display atypia and proliferation in the absence of inflammation suggest that there is a permanent change in the epithelium induced by PhIP. It is not clear if this is the result of specific mutations, or selection based upon promoter methylation induced by the inflammatory or post-inflammatory biologic niche. In our studies, we do show that there are populations of cells which down regulate GSTpi, a gene known to be down regulated in prostate epithelium via promoter methylation.

It was expected based upon previous reports that roughly half of the 65 week PhIP treated animals would have developed invasive carcinomas. None did. It may be the animals received an effectively decreased dose compared to previous publications. Storage conditions and source and grade of the PhIP reagent were identical to those previously reported, but there may still have been unanticipated degradation of the chemical or a problem with the specific production run or lot. We think this is unlikely in light of an obvious effect on the animal health (as indicated by animal weight and the 10 animals who became ill during treatment). Furthermore, we have found that PhIP is very stable in previous HPLC analyses. In the context of the histologic findings, however, additional hypotheses should be considered. Colony and housing conditions may contribute to the levels of exposure of the animals to specific bacterial flora and these can contribute to prostatic inflammation. In the context of our experiments, perhaps a cleaner or flora shifted colony resulted in decreased penetrance of the invasive cancer phenotype. Regardless of the absence of invasive cancer, it should be noted that several animals developed definitive PIN lesions, and that PIN lesions are never seen in rats or mice as spontaneous lesions in untreated or non-genetically engineered prostate. It is possible that the effective dose of PhIP in our study was higher than previous reports. Mechanistically, it may be that a higher dose would result in higher specific prostate epithelial toxicity, and that this would reduce the pool of potentially neoplastic cells. This would imply that a specific subset of cells in the prostate is susceptible to both PhIP toxicity and PhIP initiated carcinogenesis.

It also is hard to compare the reported invasive phenotypes in the literature, because few histologic images are available. In our study there were areas of profound inflammation with stromal reaction, sclerosis, and significant epithelial cytologic atypia (atypia of repair). Some of these might mimic invasion, out of the

8

context of the glandular architecture. Further study is needed to determine if the PIN lesions seen do in fact progress to invasive carcinomas. It will be useful to characterize the histomorphology of invasive carcinomas occurring in PhIP treated rats and to compare the morphology to human prostate cancers. This comparative pathology is an essential part of validating the model for further study, including pre-clinical trials.

The finding that F344 rats have Leydig cell hyperplasia and Leydig cell testicular tumors may explain the overall susceptibility of this strain to prostate tumorigenesis. One group has compared the susceptibility across common laboratory strains and measured testosterone and estradiol levels. Interestingly, the F344 rats had the second highest testosterone levels at 54 weeks, and the highest estradiol levels. Only SHR rats (spontaneously hypertensive rats) had significantly higher testosterone levels than the other strains compared, and these and ACI rats appeared to be the most sensitive to prostate neoplasia[14]. This remains an important consideration when modeling endocrine responsive tissues and cancers.

We and others have shown that PhIP is capable of forming DNA adducts, and inducing DNA mutations in the F344 rat prostate[8, 9, 15, 16]. It has generally been proposed that low levels (relative to the experimental treatment in rats) of PhIP in the Western diet might induce low levels of mutations in the human prostate epithelium that might accumulate and confer a selective advantage on epithelial cells, ultimately resulting in cancer. Other groups have proposed that PhIP is a potent endocrine hormone analog[17]. Several groups have used the rat model to test the effect of potential adduct and mutation preventing agents[8, 18-20]. This is a highly useful surrogate endpoint which can be quantified with high specificity at early time points, before morphologic changes arise. It is not precisely known that these adducts and mutations will lead to the progressive acquisition of cancer cell characteristics. In part, it was the goal of this study to try to more clearly make this association.

Meanwhile, increasing evidence suggests that prostatic inflammation and the resulting reactive processes are highly associated with cancer risk. The concept of prostatic inflammatory atrophy has been proposed to be a prostate cancer precursor, or at least to increase susceptibility to further changes resulting in prostate cancer[3, 6]. A number of molecular correlates with post-inflammatory atrophic morphology have been established, including promoter methylation and gene silencing for GSTpi and Cox2[5, 19, 21, 22]. We have shown that benign prostate lesions are sometimes accompanied by changes in apoptosis mediator proteins [22].

Our interest in PhIP administration in rodents as a model of human prostate carcinogenesis is chiefly based in clinical epidemiology. High meat and fat in the western diet, and particularly in African Americans, may influence both the development and progression of prostate cancer. We have previously

9

demonstrated the exquisite sensitivity of the prostate to PhIP. At a dose equivalent to one overcooked hamburger fed to the F344 rat, DNA adducts can be detected in the prostate[8]. Our data suggests that the PhIP treated rat will also help to characterize the role of prostate inflammation in cancer initiation. In summary, we have shown that the PhIP treated rat is a useful model of human disease suitable for testing prevention strategies targeting either DNA adduct formation or prostate inflammation.

Acknowledgments

References

[1]   Shirai T, Asamoto M, Takahashi S, Imaida K. Diet and prostate cancer. Toxicology 2002;181-182:89-94.

[2]   Gardner WA. Hypothesis: the prenatal origins of prostate cancer. Hum Pathol 1995;26:1291-2.

[3]   Gardner WA, Jr., Culberson DE. Atrophy and proliferation in the young adult prostate. J Urol 1987;137:53-6.

[4]   Smith CJ, Gardner WA, Jr. Inflammation-proliferation: possible relationships in the prostate. Prog Clin Biol Res 1987;239:317-25.

[5]   Nelson WG, De Marzo AM, DeWeese TL. The molecular pathogenesis of prostate cancer: Implications for prostate cancer prevention. Urology 2001;57:39-45.

[6]   De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am J Pathol 1999;155:1985-92.

[7]   Bogen KT, Keating GA. U.S. dietary exposures to heterocyclic amines. J Expo Anal Environ Epidemiol 2001;11:155-68.

[8]   Dingley KH, Ubick EA, Chiarappa-Zucca ML, Nowell S, Abel S, Ebeler SE, Mitchell AE, Burns SA, Steinberg FM, Clifford AJ. Effect of dietary constituents with chemopreventive potential on adduct formation of a low dose of the heterocyclic amines PhIP and IQ and phase II hepatic enzymes. Nutr Cancer 2003;46:212-21.

[9]   Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, Hasegawa R, Imaida K, Matsumoto K, Wakabayashi K, Sugimura T, Ito N. The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. Cancer Res 1997;57:195-8.

[10]   Shirai T, Cui L, Takahashi S, Futakuchi M, Asamoto M, Kato K, Ito N. Carcinogenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in the rat prostate and induction of invasive carcinomas by subsequent treatment with testosterone propionate. Cancer Lett 1999;143:217-21.

[11]   Shappell SB, Thomas GV, Roberts RL, Herbert R, Ittmann MM, Rubin MA, Humphrey PA, Sundberg JP, Rozengurt N, Barrios R, Ward JM, Cardiff RD. Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. Cancer Res 2004;64:2270-305.

[12] Park JH, Walls JE, Galvez JJ, Kim M, Abate-Shen C, Shen MM, Cardiff RD. Prostatic intraepithelial neoplasia in genetically engineered mice. Am J Pathol 2002;161:727-35.

[13] Weisburger JH, Rivenson A, Reinhardt J, Braley J, Pittman B, Zang E. On the occurrence of Leydig cell tumors in the F344 rat. Cancer Lett 2002;182:213-6.

[14] Inaguma S, Takahashi S, Ohnishi H, Suzuki S, Cho YM, Shirai T. High susceptibility of the ACI and spontaneously hypertensive rat (SHR) strains to 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) prostate carcinogenesis. Cancer Sci 2003;94:974-9.

[15] Archer CL, Morse P, Jones RF, Shirai T, Haas GP, Wang CY. Carcinogenicity of the N-hydroxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 2-amino-3, 8-dimethyl-imidazo[4,5-f]quinoxaline and 3, 2'-dimethyl-4-aminobiphenyl in the rat. Cancer Lett 2000;155:55-60.

[16] Stuart GR, Holcroft J, de Boer JG, Glickman BW. Prostate mutations in rats induced by the suspected human carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Res 2000;60:266-8.

[17] Lauber SN, Ali S, Gooderham NJ. The cooked food derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine is a potent oestrogen: a mechanistic basis for its tissue-specific carcinogenicity. Carcinogenesis 2004;25:2509-17.

[18] Imaida K, Tamano S, Kato K, Ikeda Y, Asamoto M, Takahashi S, Nir Z, Murakoshi M, Nishino H, Shirai T. Lack of chemopreventive effects of lycopene and curcumin on experimental rat prostate carcinogenesis. Carcinogenesis 2001;22:467-72.

[19] Nelson CP, Kidd LC, Sauvageot J, Isaacs WB, De Marzo AM, Groopman JD, Nelson WG, Kensler TW. Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. Cancer Res 2001;61:103-9.

[20] Schut HA, Yao R. Tea as a potential chemopreventive agent in PhIP carcinogenesis: effects of green tea and black tea on PhIP-DNA adduct formation in female F-344 rats. Nutr Cancer 2000;36:52-8.

[21] Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H. Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res 2005;65:4218-27.

[22] Gandour-Edwards R, Mack PC, Devere-White RW, Gumerlock PH. Abnormalities of apoptotic and cell cycle regulatory proteins in distinct histopathologic components of benign prostatic hyperplasia. Prostate Cancer Prostatic Dis 2004;7:321-6.

Figure Legends

Figure 1. Quantified changes in PhIP and Control Prostate Glands. Overview of histologic changes in the PhIP treated (solid bars) and control (hatched bars) rats. The median (horizontal line), 25th to 75th percentile (column boxes) and range (tail bars) of percent of prostate glands involved is plotted for groups at each time point (25, 45, and 65 weeks of age). An unpaired nonparametric t-test was applied to the groups at each time and the resulting p values are shown above the data bars. *The t-test could not be applied to the PIN data because all of the values in many of the columns were zero.* The percentages of the glands showing inflammation is shown in panel A., atrophy in panel B, atypia in panel C, and prostatic intraepithelial neoplasia in panel D.

Figure 2. Atypical Proliferations Arise in Inflamed Prostate. Within areas of markedly inflamed prostate, foci of proliferative epithelium are seen. Low power view of the dorsal and lateral prostate (A) shows a segmental area of inflammation (*Infl*) and another area of atrophy (*Atr*). The urethra (*U*) is at the top right, and the box indicates the location of the high magnification seen in (B). The high magnification shows epithelial proliferation with cellular loss of polarity and cytologic atypia.

Figure 3. PhIP Induces Prostate Epithelial Proliferation. The PHIP treated prostate shows abnormal proliferation with mitoses indicated by the arrows as compared to the control vehicle treated animal on the right. Both panels are ventral prostate glands from rats. Images from formalin fixed paraffin embedded tissue 4 micron sections stained with hematoxylin and eosin, obtained on a Zeiss Axioskop equipped with the Zeiss AxioCam digital camera using the Axiovision acquisition software. Scale bars indicate actual size (lower right of each panel).

Figure 4. PIN arises in atrophy in PHIP treated rat prostate. Within the atrophic glands, occasional areas of proliferation with loss of polarity and cells forming cribriform spaces and Roman bridges are seen. The high proliferation of these areas is confirmed by the Ki67 immunostain (B) showing a marked increase in percent cell staining (nuclear stain) in these regions.

Figure 5. Atypical cells in PHIP treated rat prostate show loss of GSTp1, Cox2 is roughly unchanged. The atypical cells shown (arrows in panel A) have abundant cytoplasm and nuclei with single prominent nucleoli. Basal cells can be seen interspersed among these cells and are highlighted by the GST immunostain *(B)*. Cox2 (C)may be slightly reduced in the same cells, but is detected by the immunostain.

Figure 6. F344 Rats Develop Leydig Cell Hyperplasia. Rats in both the control and PhIP treated groups developed Leydig cell hyperplasia with high penetrance. Patchy areas of Leydig cell hyperplasia, varying in size were seen in a majority of animals at the 65 weeks of age time point. This example shows a relatively large patch of Leydig cells (*Ley*) adjacent to two seminiferous tubules (*Tub*).
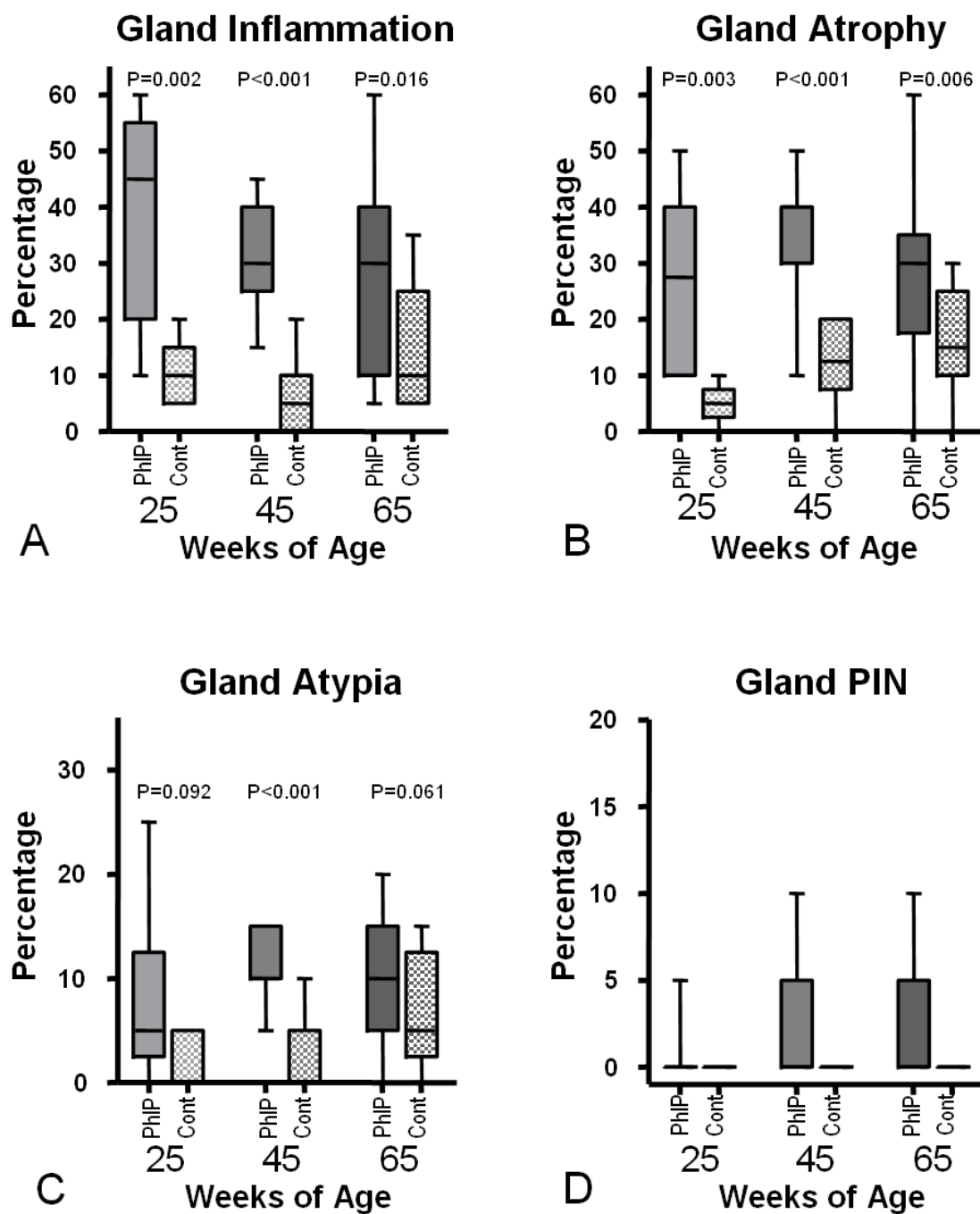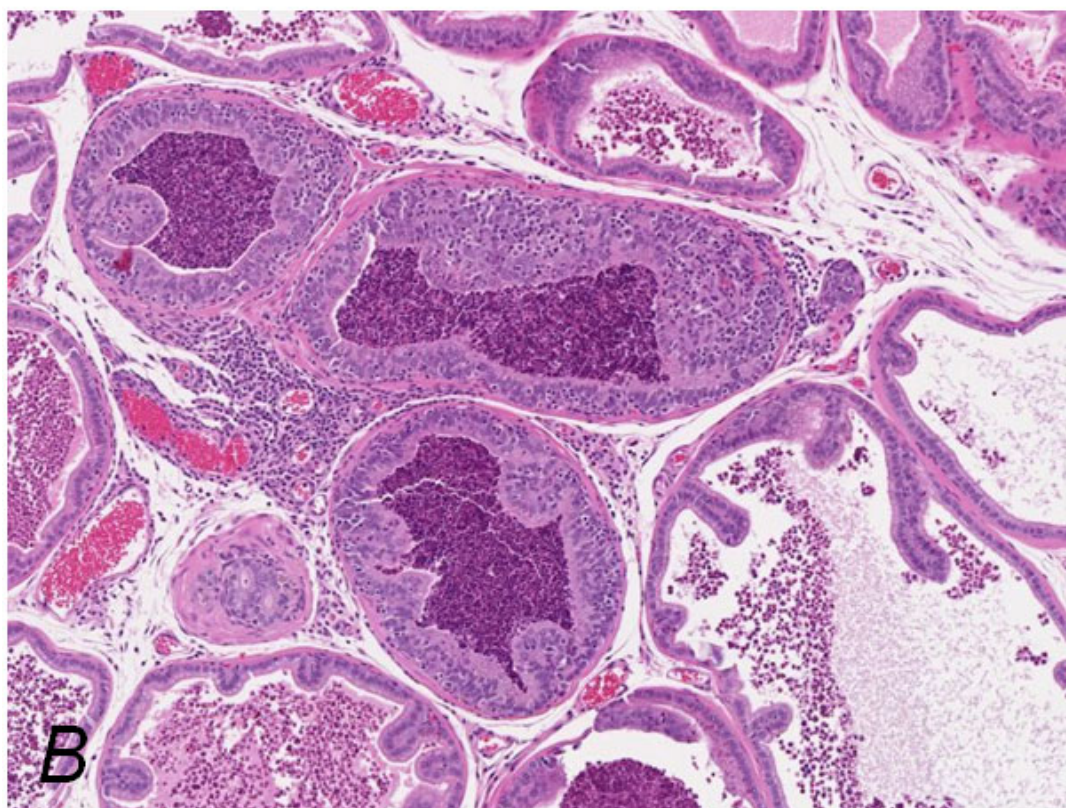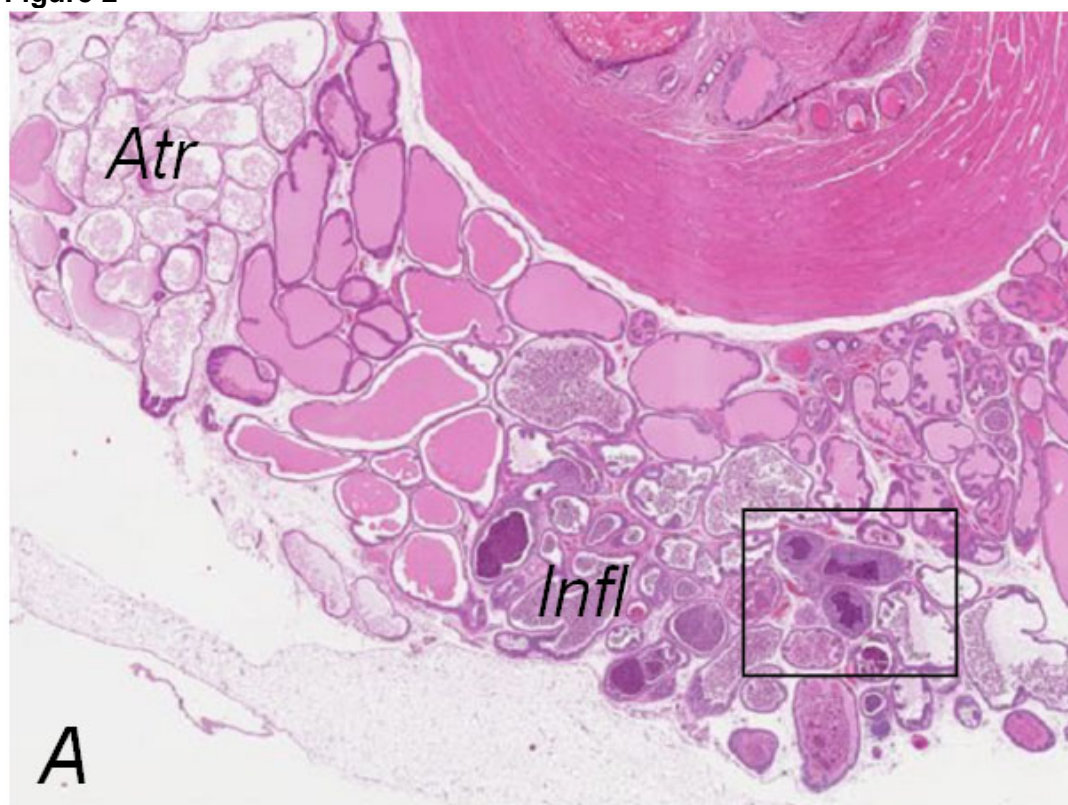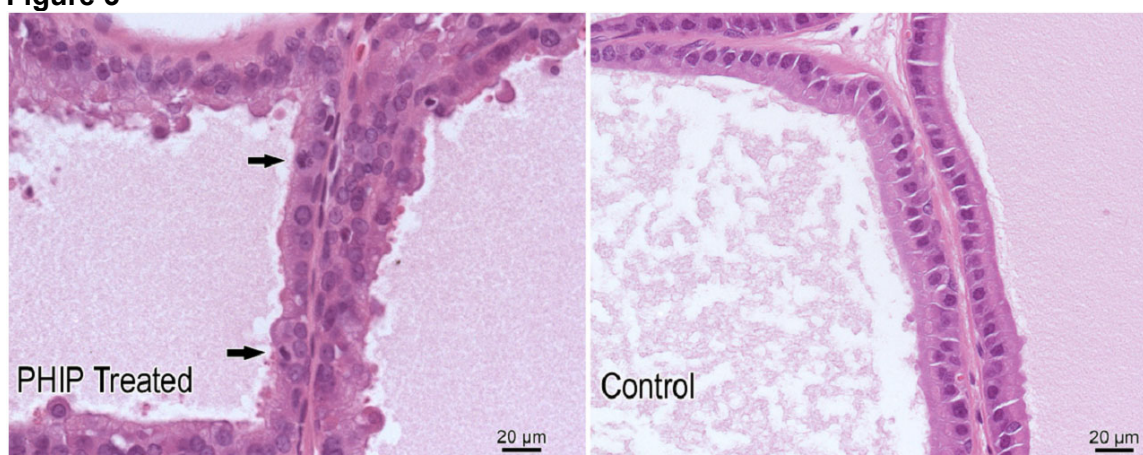
Figure 1

Gland Inflammation

Percentage

P=0.002   P<0.001   P=0.016

PhIP Cont PhIP Cont PhIP Cont
25   45   65

A   Weeks of Age

Gland Atrophy

Percentage

P=0.003   P<0.001   P=0.006

PhIP Cont PhIP Cont PhIP Cont
25   45   65

B   Weeks of Age

Gland Atypia

Percentage

P=0.092   P<0.001   P=0.061

PhIP Cont PhIP Cont PhIP Cont
25   45   65

C   Weeks of Age

Gland PIN

Percentage

PhIP Cont PhIP Cont PhIP Cont
25   45   65
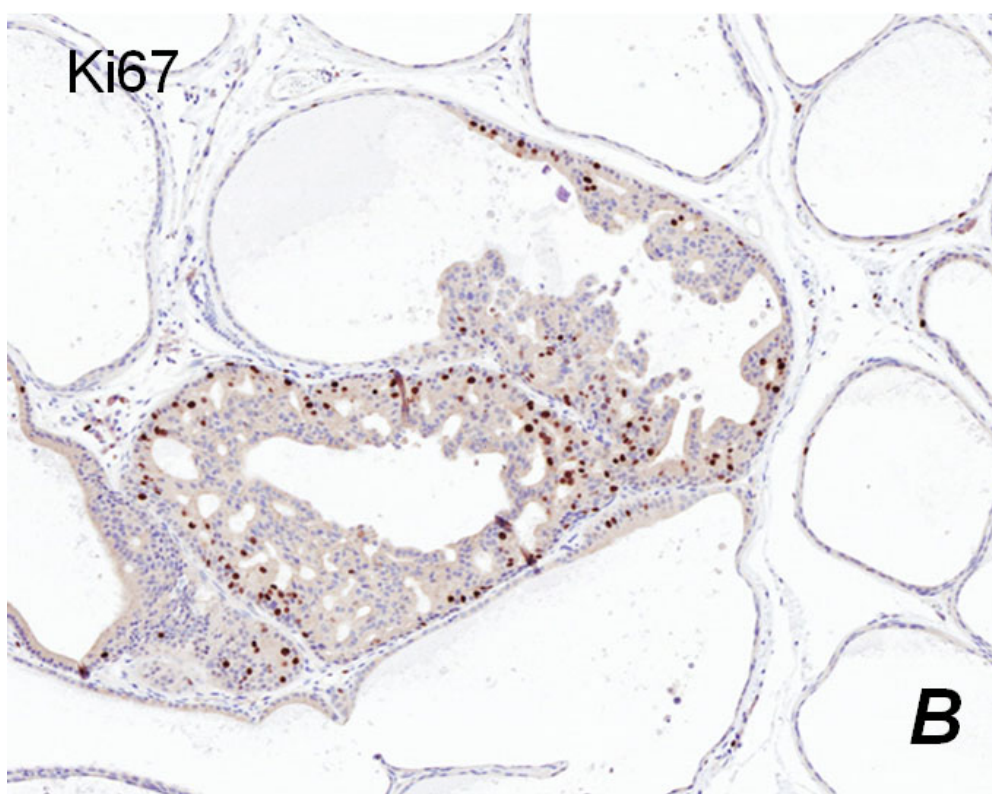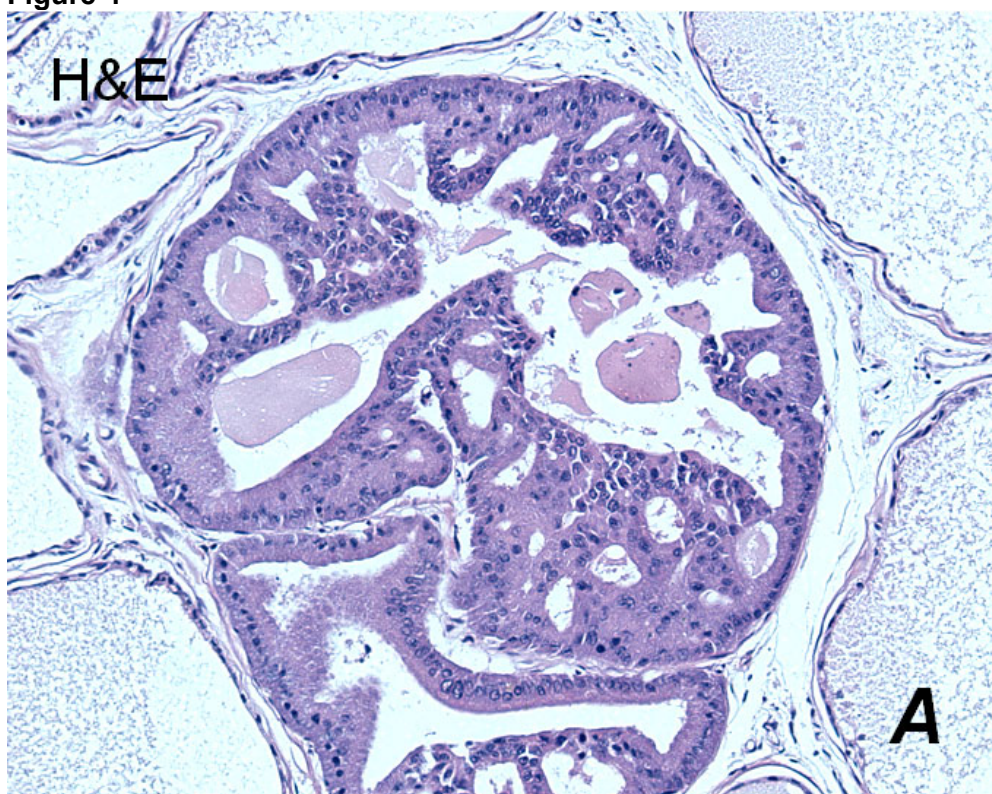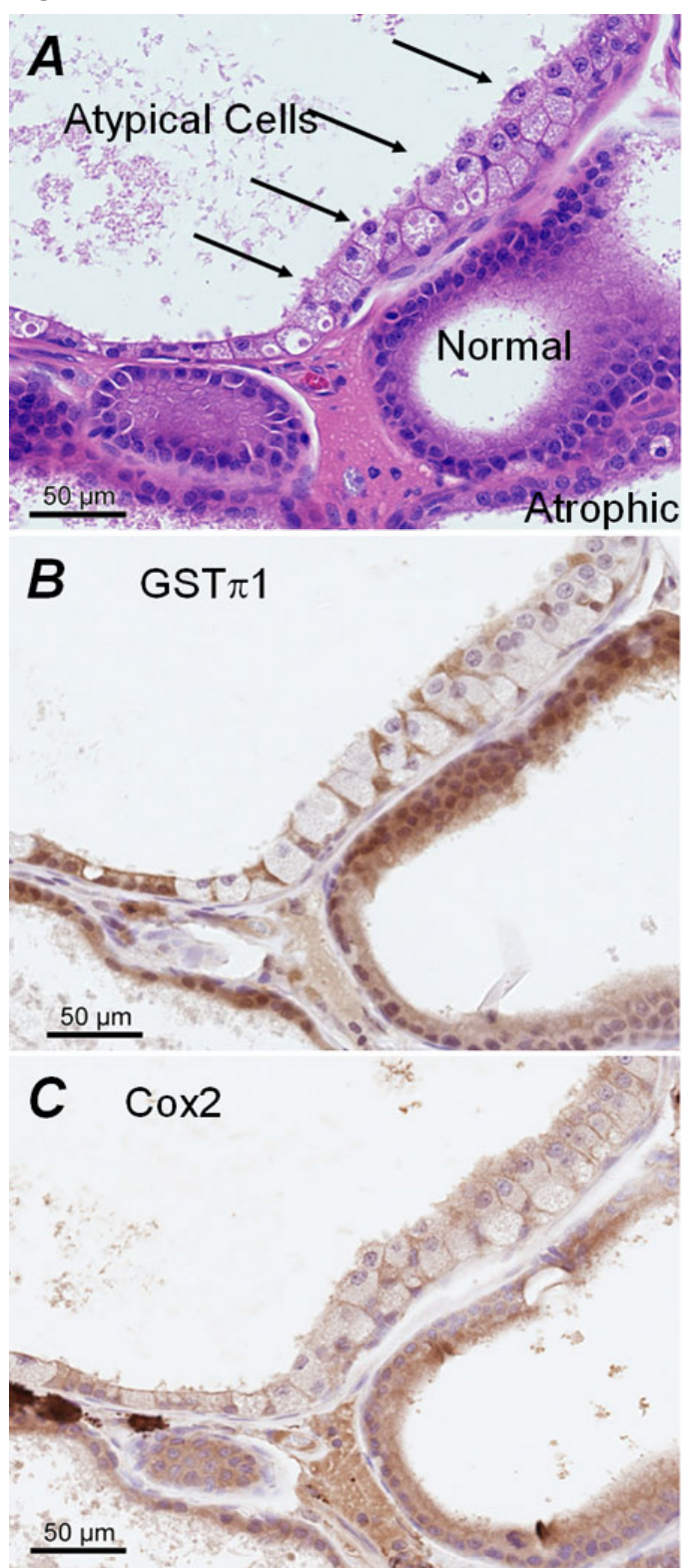
D   Weeks of Age

15

Figure 2

Figure 3

PHIP Treated

20 μm

Control

20 μm

Figure 4

Figure 5

Figure 6